Association between the A1166C polymorphism of the angiotensin II receptor type 1 and progression of chronic renal insufficiency

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ABSTRACT: *Background:* **In some studies genetic variation in the renin-angiotensin-aldosterone system (RAAS) has been associated with hypertension and rapid progression of renal insufficiency to end-stage renal disease (ESRD). Most of these studies do not take into account covariables influencing progression. We studied retrospectively the role of angiotensinogen (AGT) M235T, angiotensin converting enzyme (ACE) insertion/deletion (I/D), angiotensin II type 1 receptor (AT1R) A1166C, aldosterone syntase (CYP11B2) -344C/T and intron 2 W/C polymorphisms in conjunction with clinical and biochemical covariables on the rate of progression of renal insufficiency in a group of patients with ESRD of various etiologies.**

Methods: **Genotyping was performed by polymerase chain reaction (PCR) in 104 ESRD patients (62 males and 42 females), aged 64 ± 14 years (mean ± SD) with mean initial serum creatinine of 2.6 ± 1.1 mg/dL and a mean time to reach ESRD of 52 ± 38 months.**

Results: **The univariate analysis showed that there was a significant difference in the values of the slopes among the AT1R A1166C polymorphism genotypes: AA -4.87 ± 0.22, AC -5.09 ± 0.65 and CC -5.52 ± 0.66 ($p<0.05$). None of the remainder polymorphisms showed significant association with progression. Stepwise multiple regression analysis including all the clinical, biochemical and genetic variables showed that only systolic blood pressure (SBP), serum PTHi and AT1R genotype were independently associated with the rate of progression, excluding the other variables from the model.**

Conclusions: **These results indicate that susceptibility to faster progression to ESRD is associated with the AT1R A1166C polymorphism. This association remains significant after adjustment for relevant covariates, highlighting the importance of analyzing genetic risk factors in the context of clinical and biochemical variables.**

Key words: *ACE, CYP11B2, AT1R, Angiotensinogen, Genes, Kidney, Progression, Polymorphism*

INTRODUCTION

Clinical studies have demonstrated that beyond a certain degree of nephron number reduction, chronic renal insufficiency gradually progresses towards end-stage renal disease (ESRD), even when the causative disease has been cured or improved. The mechanisms underlying the progression of renal disease have been intensely studied in recent years. The rate of progression of chronic renal insufficiency appears to be relatively constant in a given individual regardless of the etiology. Brenner et al have proposed that nephron number loss as a consequence of renal disease is followed by compensation by surviving nephrons to normalize single nephron glomerular filtration rate (1). This compensating process tends to be detrimental because hypertrophy and hyperfiltration of surviving nephrons induces glomerulosclerosis and additional

reduction of nephron mass initiating a self-perpetuating cycle that ends in kidney failure. Glomerular hemodynamics has an initial crucial role in the compensatory response of surviving nephrons, and these processes are regulated mainly by the renin-angiotensin-aldosterone system (RAAS). RAAS is involved in the regulation of arterial blood pressure (BP). Beyond its glomerular hemodynamic effects RAAS exerts a non-hemodynamic action through induction of tissue growth and fibrosis; therefore, this system plays an important pathogenic role in the development and progression of renal damage (2).

Hypertension is a major contributor in the progression of renal failure in renal disease patients (3) both with and without proteinuria. The use of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor type 1 antagonists have been shown to have protective renal effects because of their ability to reduce urinary protein excretion and to retard the progression of renal failure, effects that seem to be beyond their BP reducing effect (4).

Some studies have shown a strong association between family history of ESRD and increased risk of ESRD, suggesting a genetic component of the disease (5). In addition, there is evidence of a genetic susceptibility to ESRD progression since among patients with treated hypertension some develop hypertensive nephropathy progressing to ESRD, whereas others remain stable regardless of BP control and antihypertensive treatment (6). Therefore, a search for the association between genetic polymorphisms in candidate genes and development of ESRD has been recently initiated.

Polymorphisms of RAAS genes (ACE, angiotensinogen, AT1 receptor and aldosterone synthase) have been described, and have been associated with a number of phenotypes of cardiovascular relevance, i.e. salt-sensitivity and upregulation of this homeostatic system (7, 8). Some of these polymorphisms have been associated with essential hypertension (9-12), target organ damage (13) and ESRD progression in some studies (14-19), but not in others (20-23). However, in most genetic studies, clinical and biochemical factors relevant to the renal outcome have not been taken into account. It is probable that progression is a multifactorial process, involving several genes in conjunction with known clinical risk factors.

We have retrospectively analyzed the role of polymorphisms of RAAS genes angiotensinogen (AGT) M235T, ACE insertion/deletion (I/D), angiotensin II type 1 receptor (AT1R) A1166C, aldosterone syntase (CYP11B2)-344C/T and intron 2 conversion (W/C), in conjunction with clinical and biochemical parameters, in a multivariate analysis on the progression of renal insufficiency in a group of patients with ESRD of several etiologies.

SUBJECTS AND METHODS

Patients and clinical data

The study evaluated patients undergoing maintenance dialysis at the Division of Nephrology of the University Hospital Clinic of Barcelona and two peripheric dialysis centers linked to the hospital. Clinical information and biochemical parameters were retrieved retrospectively from hospital records and from a computerized biochemical database. Only those patients were selected who had at least four recorded serum creatinine measurements spanning more than one year of follow-up, pre-ESRD to calculate properly the slope of reciprocal creatinine versus time (24). The study consisted of 104 patients fulfilling the criteria. The hospital ethical committee approved this study. Consent was obtained from all patients. The risk factors analyzed for progression were: age, sex, smoking status, etiology of renal insufficiency, BP, hyperuricemia, dyslipidemia, serum calcium, serum phosphorus and treatment with ACE inhibitors and were recorded at the time of renal insufficiency diagnosis. The age of onset of end-stage renal failure was considered the age at which long-term renal replacement therapy was initiated.

As a control group, 133 healthy subjects from the hospital staff, and patients from the Ophthalmology Department were analyzed with the following criteria: age between 25 and 75 years; absence of nephropathy or renal failure, diabetes mellitus or cardiovascular diseases (including hypertension, myocardial infarction or stroke). The group consisted of 72 males and 61 females aged 60 ± 13 years with a mean systolic blood pressure (SPB) and mean diastolic blood pressure (DBP) of 117 ± 11 and 69 ± 7 mmHg, respectively.

Molecular studies

Genomic DNA was isolated from peripheral-blood lymphocytes by the salting out procedure, as described previously (25).

The ACE I/D polymorphism was amplified with the flanking primers, as described previously (26), separated on a 2% agarose gel, and visualized by ultraviolet transillumination. The 490-bp and 190-bp products correspond to respectively the I and D alleles. Some patients, erroneously considered DD because of the preferential amplification of the smaller D allele, were reamplificated with a insertion-specific primer pair and showed a 335-bp product corresponding to the I allele (27). The resulting genotypes were DD, DI and II.

Angiotensinogen M235T genotype was determined by PCR amplification using allele-specific primers, as described previously (28). PCR products were digested by SfNal (New England Biolabs, Beverly, Mass, USA),

electrophoresed in 10% polyacrylamide gel and visualized by staining with ethidium bromide. Amplification of the T allele resulted in a 303-bp DNA fragment and digestion of the M allele resulted in a 266-bp DNA fragment. The resulting genotypes were MM, MT and TT.
To determine the AT1R A1166C genotype, PCR amplification was performed under conditions described previously (11). PCR products were digested by Dde1 (Toyobo, Osaka, Japan), electrophoresed in 2% agarose gel and visualized by staining with ethidium bromide. Amplification of the A allele resulted in a 546-bp DNA fragment and digestion of the C allele resulted in a 435-bp DNA fragment. The resulting genotypes were AA, AC and CC.
For the analysis of the CYP11B2 -344C/T polymorphism, PCR amplification was performed under conditions described previously (29). PCR products were digested with Hae III for 2 hours at 37 °C. The -344T allele lacks the Hae III site of the -344C allele, where the PCR product (125-bp) was cut into two fragments of 97-bp and 56-bp. Analysis of the intron 2 polymorphism was carried out using allele-specific primers and PCR conditions, as described previously (30). Amplified products were electrophoresed in a 2% agarose gel and visualized by staining with ethidium bromide. Oligonucleotides localized in intron 3 of CYP11B1 and CYP11B2 and those localized in intron 2 of CYP11B1 detected the presence of the converted (conv) allele. DNA from subjects known to carry the normal (wt) and converted alleles by sequencing were amplified as positive controls. The resulting genotypes were WW, WC and CC.

Statistical analysis

Renal failure progression was assessed by means of simple linear regression analysis, using a single straight line to model the relation of the reciprocal creatinine (1/Cr) against time. For each patient the regression coefficients were calculated by ordinary least squares (OLS). The slope so obtained gave an estimation of the rate of change in renal function, and was then used as a summary measure of a patient's response profile (31).
To use the slope values as a response variable in further analysis, the distribution of these data were examined graphically and with the Kolmogorov-Smirnov test. A logarithm transformation was finally applied to the absolute value of the slopes to correct for asymmetry.
Clinical and genetic variables gathered to explain renal failure progression were tested by univariate analysis before being included in the multivariable model. For clinical dychotomic data (sex, smoking, dyslipemia, hyperuricemia, diabetes mellitus and treatment with ACE-inhibitors) two sample Student's t-tests were performed to compare the log of the slopes between groups. Differences among the etiologies of renal disease were tested by one-way analysis of variance (ANOVA). Among continuous data (age, calcium, phosphorus, serum PTH, hematocrit, serum potassium, SBP and DBP), candidates included in the model were selected by calculating Pearson's and Spearman's correlation coefficients.
Genotypic frequency distribution for single polymorphic sites (AS344, WTCONV, AT1R, ACE-ID and AGT235) in this sample was compared with control samples by chi-square test. Hardy-Weinberg disequilibrium was also tested for each polymorphic site using a chi-square statistic.
Differences in the log of the slopes among genotypes of single sites were tested by one-way ANOVA. Combined genotypes that took into account two polymorphics sites were explored, but not tested because some subgroup sizes were small or null.
The multivariate model was constructed following a forward purposeful selection strategy. To start with, the continuous variables that correlated significantly with the log of the slope were selected as candidates to be in the model. Genetic information for sites A1166C and M235T were proposed with the stepwise regression procedure included in the regression model. To avoid the effect of ordered coding, these variables were included as fixed factors in a general linear model and tested by ANOVA. Two options were examined: maintaining three genotypes per site and reducing the three groups into two. Finally, clinical data with marginal significance in the univariate analysis were also included in the stepwise procedure. ANOVA was used when the baseline characteristic was a continuous analysis of variance. Chi-square analysis was used when comparing allele frequency of genotypes between the groups. Data are means ± SD, with range indicated in parenthesis when appropriate. Values of $p<0.05$ were considered as significant.

RESULTS

Characteristics of participants

The 104 patients included 62 men and 42 women aged 64 ± 14 years. The mean SBP and DBP were 157 ± 21 (120-200) and 87 ± 9 (70-120) mmHg, respectively. The mean creatinine level at presentation was 2.6 ± 1 mg/dL and the average time to reach hemo-dialysis was 51.6 ± 38 (12-244) months. The follow-up values of the analytical parameters were: serum calcium 8.9 ± 0.9 (5.6-10.8) mg/dL, serum phosphate 4.7 ± 1.4 (2.7-9.2) mg/dL, serum intact PTH 286 ± 241 (11-1280) pg/mL, hematocrit 33 ± 6 (22-49) %, serum K 4.7 ± 0.7 (3.3-7.1) mEq/L, total cholesterol 225 ± 58 (100-

402) mg/dL and triglycerides 157 ± 86 (51-655) mg/dL. The clinical conditions registered were smoking (n=36, 35%), hyperlipemia (n=31, 30%), hyperuricemia (n=26, 25%) and diabetes mellitus (n=30, 29%; 7 with type 1 and 23 with type 2). The following renal disease etiologies were considered: nephroangiosclerosis (n=35), diabetes mellitus (n=21), specified and unspecified glomerulonephritis (n=14), polycystic kidney disease (n=10), chronic interstitial nephritis (n=5) and unknown (n=19). For comparisons, etiologies were grouped into glomerular disease (glomerulonephritis and diabetic nephropathy) and tubulointerstitial disease.

Analysis of genotypes

The frequencies of the different genotypes studied did not deviate from the Hardy-Weinberg equilibrium in patients and in controls except for CYP11B2 WT/CONV in controls, p=0.04. Comparison of genotype distribution between ESRD patients and controls is shown in Table I. The genotype distribution of the CYP11B2 WT/CONV polymorphism was significantly different between patients and controls, possibly due to deviation in the controls. There were no significant differences in genotype frequencies of CYP11B2 -344 C/T, AT1R A1166C, ACE I/D and AGT M235T polymorphisms between patients and controls.

Genetic polymorphisms were analyzed for their relationship to the rate of progression of renal disease (Tab. II). With respect to AT1R A1166C polymorphism, progression to ESRD was more rapid in patients with AA genotype of AT1R (slope= -4.87), than AC genotype (slope= -5.09) or CC genotype (slope= -5.53), p=0.04. Patients homozygous for the AGT M235T polymorphism (MM and TT) showed faster rates of progression of renal failure (respectively -4.82 and -4.91) than heterozygous (-5.23) (p=0.02) (Tab. II). There were no significant differences in plasma creatinine at presentation, SBP or serum intact PTH levels (data not shown) among AT1R A1166C polymorphism genotypes. For the remainder of RAAS polymorphisms there were no significant differences in the rate of progression among genotypes.

Clinical and biochemical factors were also analyzed in relation to rate of progression of renal failure. As anticipated, patients in the subgroup of glomerular diseases progressed faster (slope -4.74 ± 0.67) than the patients with tubulointerstitial disorders (slope -5.14 ± 0.62) (p=0.007).

Table III shows the categorical clinical and biochemical parameters that were selected for the analysis. When considered individually, none of these parameters were associated with the rate of progression of renal insufficiency in our patient sample. Possibly due to the retrospective nature of the study, only 28% of the patients had been treated with ACE inhibitors and the presence or absence of this treatment did not affect the rate of progression. Table IV contains the continuous clinical and biochemical parameters and shows that only SBP and serum intact PTH correlated with the slope of the inverse of creatinine versus time (R=0.31, R=-0.34, respectively, p=0.004).

In the multivariate model, only SBP, serum intact PTH

TABLE I - DISTRIBUTION AND FREQUENCIES OF GENOTYPES IN PATIENTS AND CONTROLS

Gene/ Polymorphism	Patients (N=104)			Controls (N=133)			P
CYP11B2/ 344C/T	CC 16 (15.4%)	CT 58 (55.7%)	TT 30 (28.9%)	CC 32 (24%)	CT 53 (40%)	TT 46 (36%)	0.052
CYP11B2/ WT/CONV	WW 24 (23%)	WC 62 (60%)	CC 18 (17%)	WW 49 (37%)	WC 51 (38%)	CC 33 (25%)	0.005
ACE/ I/D	II 17 (16%)	ID 45 (43%)	DD 42 (41%)	II 15 (15%)	ID 48 (47%)	DD 39 (38%)	0.855
AT1R/ A1166C	CC 5 (5%)	AC 44 (42%)	AA 55 (53%)	CC 11 (9%)	AC 66 (50%)	AA 54 (41%)	0.165
AGT/ M235T	TT 39 (37%)	TM 37 (36%)	MM 28 (27%)	TT 32 (32%)	TM 47 (46%)	MM 23 (22%)	0.292

Frequencies are indicated in parenthesis; χ^2 between patients and controls.

and AT1R A1166C polymorphism were independently associated with the rate of progression with at least 95% confidence (Tab. V). The standard scores show that the variables serum intact PTH and SBP contributed 18% and the genetic variable AT1R A1166C polymorphism contributed 10% to the total variability of the slope.

Discussion

We analyzed five RAAS gene polymorphisms for association with the progression of chronic renal failure to ESRD. RAAS genes are potential etiological candidates for progression to ESRD because of their effects on renal tissue growth and fibrosis (2, 32).

RAAS genetic polymorphism has been associated with organ damage in essential hypertension (ACE I/D polymorphism, AGT M235T polymorphism and AT1R A1166C polymorphism), the patients with the combination of genotypes ACE DD + AT1R CC + AGT TT being at highest risk of hypertensive target organ damage (33).

Wang et al analyzed three gene polymorphisms (ACE I/D, CYP11B2 -344CT and α-adduccin Gly460Trp) for the presence of mild renal insufficiency in a population sample. Allele D of ACE gene was associated with an increased risk of mild renal dysfunction. A combined gene analysis showed that renal function was slightly but consistently impaired when both the ACE D and α-adducin Trp alleles were present. In this study BP, antihypertensive treatment, diabetes, and urinary protein excretion were also evaluated and did not affect the results, even when hypertensive patients and women on hormonal therapy were excluded (34).

Some studies have examined the role of RAAS polymorphisms in renal disease development or progression in individual nephropathies. In these cases, there

TABLE II - RATE OF PROGRESSION OF RENAL INSUFFICIENCY AMONG GENOTYPES OF CANDIDATE GENES POLYMORPHISMS

Gen / Polymorphism	Slope*	P-value
CC (16)	-4.72 ± 0.52	0.201
CYP11B2 344CT CT (58)	-5.05 ± 0.75	
TT (30)	-5.04 ± 0.51	
WW (24)	-4.82 ± 0.81	0.245
CYP11B2 WT/CONV WC (62)	-5.08 ± 0.64	
CC (18)	-4.94 ± 0.48	
II (17)	-5.22 ± 0.44	0.296
ACE I/D ID (45)	- 4.92 ± 0.75	
DD (42)	-4.99 ± 0.62	
CC (5)	-5.53 ± 0.22	0.04
AT1R A1166C AC (44)	-5.09 ± 0.66	
AA (55)	-4.87 ± 0.66	
TT (38)	-4.82 ± 0.62	0.02
AGT M235T TM (36)	-5.24 ± 0.59	
MM (28)	-4.91 ± 0.74	

*Data are means ± SD, *slope is log of the slope of 1/creatinine versus time, ANOVA.*

TABLE III - TWO-GROUPS COMPARISON OF THE LOG OF THE SLOPE BY STUDENT'S T-TESTS

Factor	n^1 / n^2	95% C.I.	P-value
Sex	62 / 42	-0..354 – 0.171	0.490
Smoking	36 / 68	-0.214 – 0.329	0.676
Dyslipemia	31 / 73	-0.255 – 0.310	0.846
Hyperuricemia	26 / 78	-0.571 – 0.016	0.063
Diabetes mellitus	30 / 74	-0.052 – 0.511	0.109
ACEI treatment	29 / 75	-0.191 – 0.384	0.507

n^1 accounts for number of patients with risk factor and n^2 number of patients without.

TABLE IV - ASSOCIATION RESULTS BETWEEN THE LOG OF THE SLOPE AND CLINICAL CONTINUOUS VARIABLES

FACTOR	Pearson's r	P-value	Spearman's	P-value
Age	-0.139	0.160	-0.162	0.101
Calcium	-0.143	0.148	-0.190	0.054
Phosphate	0.032	0.747	0.059	0.553
Intact PTH	-0.340	<0.001	-0.281	0.004
Hematocrit	0.037	0.707	0.030	0.763
Serum K	-0.046	0.642	-0.054	0.585
SBP	0.313	0.001	0.277	0.004
DBP	0.088	0.374	0.012	0.905

P values are two-tailed. N = 104.

TABLE V - POINTWISE AND 95% INTERVAL ESTIMATES OF THE REGRESSION COEFFICIENTS FOR THE MULTIVARIATE REGRESSION MODEL OBTAINED BY STEPWISE SELECTION

Variable	B 95%	CI P	P-value
Serum intact PTH	-0.00091	-0.001 - 0.000	0.001
SBP	0.0077	0.002 - 0.013	0.006
AGT M235T	-0.322	-0.554 - -0.090	0.007
AT1R A1166C	0.267	0.042 - 0.491	0.021
Hyperuricemia	0.278	0.012 - 0.543	0.041

is great disparity in the results depending on the sample and disease studied. No conclusions can be made about the role of RAAS polymorphisms in disease severity in polycystic kidney disease (21) and IgA nephropathy (22). In the case of diabetes, RAAS polymorphisms, mainly ACE I/D appear not to be associated to diabetic nephropathy (35), but seem to predict progression in type I diabetes (18, 19). Overall, the benefits of ACE inhibition in slowing the progression of chronic renal insufficiency in nephropathies of divergent etiologies (36) suggest that RAAS contribution to progression may not be disease-specific, because it would act on the common mechanisms of disease progression. However, this hypothesis will have to be confirmed in large clinical trials.

Our study, and others, included several types of nephropathies. Previous studies have evaluated the association between RAAS polymorphisms and progression of renal insufficiency secondary to a variety of diseases. In this way, Lovati et al analyzed three polymorphisms (AGT M235T, ACE I/D and CYP11B2 -344TC) and found an association only between DD genotype and faster progression to ESRD in the total sample. In the subgroup of glomerulonephritis the association was stronger when DD genotype was evaluated in the subgroup of AGT MM homozygotes. However, in this study clinical risk factors for progression were not evaluated, with the exception of BP and diabetes mellitus (17). Mc Laughin et al evaluated the effects of ACE I/D polymorphism in renal failure progression, and found an association between DD genotype and a faster rate of progression (14). However, when patients were separated into two groups, tubulointerstitial diseases and glomerular diseases, this association was present only in patients with glomerular pathology. As in Lovati's study no clinical risk factors for progression were evaluated. The largest prospective study evaluating the influence of polymorphisms on disease progression was a *post-hoc* study of the REIN project, which included non-diabetic proteinuric nephro-pathies (23). In this study, renal disease progression was independent of the ACE I/D polymorphism. In our patients, we were also unable to find an association between the D allele and progression.

A significant correlation between the rate of progression of renal failure and the A1166C polymorphism was found in our patients with faster progression for AA genotype ahead of AC or CC genotypes. This association has not been previously described, and contrasts with other studies that correlate allele C of ATR1 gene with hypertension (11). The explanation for this apparent contradiction could be because hypertensive nephropathy habitually progresses to ESRD slower than other nephropathies. As demonstrated by this study, patients with nephroangiosclerosis showed a slow rate of progression when compared to patients with glomerular diseases. AT1R is related to faster progression with a gene dose effect, i.e. patients with AA genotype progress the fastest to ESRD, with CC the lowest, and with AC showing an intermediate effect.

In the case of AGT polymorphism M235T, homozygous patients (MM and TT) showed faster progression of renal failure than MT patients, and at present there is no explanation for the latter finding.

Another benchmark from this study is the relevance of evaluating risk factors concomitantly with genetic factors, as is demonstrated by the correlation found between levels of SBP and serum levels of intact PTH and the rate of progression. At this point it is important to perform a multiple regression analysis to search for the independent contribution of each risk factor to the rate of progression.

There are several reasons for discrepancies in the genetic studies of progression in play. Firstly, the genetic basis of renal failure progression can be genetically complex, being likely that several genes contribute in conjunction, with individual genes showing quantitative small effects that are difficult to detect or confirm. Secondly, the diversity of the study population can affect the results of genetic associations.

In conclusion, this study demonstrates an association between the AT1R gene A1166C and the CYP11B2 -344CT polymorphisms with a faster progression to ESRD. After adjustment for relevant covariates only the A1166C polymorphism remains in the multivariate model, highlighting the importance of analyzing risk factors in the context of demographic, clinical and biochemical variables. However, large prospective studies will be necessary to assess the role and relative contribution of different RAAS polymorphisms in progression of chronic renal failure.

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